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(54) Title: IMPROVED STREPTOMYCES PROMOTER

(57) Abstract

The present invention is a DNA sequence comprising a *Streptomyces thiostrepton* promoter (*tipA*) wherein a base, preferably guanine, is inserted between positions -50 and -75, preferably positions -62 and -63. This promoter is stronger than the wild type and ultimately increases transcription initiation and protein expression.

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IMPROVED STREPTOMYCES PROMOTER

The present invention relates to a Streptomyces promoter. More specifically, the present invention is an improved promoter which is useful to ultimately increase expression and efficiency of a gene.

5 A promoter is a DNA sequence that promotes gene transcription to produce a messenger RNA and is recognized by RNA polymerase, which the promoter directs to the correct transcriptional start site. The E. coli promoter region, which is about 50 bases long, includes a common sequence (Pribnow box) of about 6 bases located upstream (in the 5' direction) from the site at which RNA synthesis begins. Five to 10 eight bases separate the Pribnow box from the initiation site, so that it centers around the -10 position. Not all E. coli promoters have exactly the same sequence of bases in 15 this region, but a sequence similar to TATAAT is typically found. Another consensus sequence, TTGACA, centers around -35; this region is also critical for the accurate and rapid initiation of transcription for most bacterial genes (see Darnell *et al.*, 1986, Molecular Cell Biology, Scientific American Books Inc.). It has been noted that the -10 and -35 regions, as well as the spacing between them, are "major determinants of 20 promoter strength." Because of the conserved regions, the RNA polymerase appears to make direct contact with the two regions. Consequently, "most mutations that affect promoter strength map to the strongly conserved bases in the -10 or -35 region or 25 change the spacing between them" (Jansen, *et al.*, 1985, Microbiology, Meetings for the American Society for Microbiology, American Society for Microbiology, Washington, D.C., pages 392-396 [1985]).

Mutations in the promoter may effect initiation. It has been shown that 30 changes, deletions, or insertions in the promoter region may effect the way that RNA polymerase binds to the promoter. The ultimate result may be that more or less mRNA is produced. For example, Mandecki *et al.*, 1982, Nucleic Acid Research 10:903-912, show that a two base pair insertion between the -10 and -35 regions lowered the promoter activity to 15% of the wild type; Post *et al.*, 1978, Cell 15:231-236, show that a point mutation six bases upstream from the *in vitro* transcription site, which changed the Pribnow box, was important in the expression of an operon; and Youderian *et al.*, 1982, Cell 30:843-853, show that specific decreases in the homology of a specific promoter with its consensus promoter sequence resulted in either a severe or a mild reduction in promoter activity (see Watson *et al.* Molecular Biology of Gene 4th edition Benjamin/Cummings Publishing Company, 1987). (Also see Botstein, 1972, Virology 35 49:268-282, and Siebenlist *et al.*, 1980, Cell 20:269-281.)

Since promoters are known to effect ultimate gene expression, it would advantageous to increase the strength of promoter by manipulating the promoter sequence. That is the focus of the present invention, which is detailed and described more fully below.

5 The present invention is a DNA sequence comprising a Streptomyces promoter, wherein a base is inserted into the base sequence between positions -50 and -75, to increase transcription initiation. Preferably, the base insertion is made between -60 and -65, more preferably between positions -62 and -63. Preferably the base is guanine. Preferably, the DNA sequence is regulated by thiostrepton and is operable in
10 Streptomyces. Preferably, the DNA sequence will hybridize to the following DNA sequence: GCGGC_nCTCACGGCGTGGCA. More preferably, the DNA sequence is shown in Figure 1. This improved DNA sequence may occur in the chromosome or may be in an expression vector. Preferably, the invention also includes a method for producing a protein using the DNA sequence detailed above.

15 The present inventor has made the surprising discovery that a base insertion between positions -62 and -63 of the Streptomyces thiostrepton promoter (tipA), increases promoter strength so that protein expression is increased 5-fold (while maintaining the regulatory aspects of the promoter). This is further surprising because the position of the mutation is outside the commonly recognized sequences for the promoter, i.e., near the -10 and -35 regions.

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Figure 1 is the DNA sequence of the tipA wild type and mutant promoters.

Figure 2 is a diagram of the construction of pHC-1 and pHC-2.

Figure 3 is a circular map of pHC-2.

25 A promoter can be induced by a compound. In Streptomyces, thiostrepton is one such compound. Thiostrepton is an antibiotic which inhibits translation. However, it has been reported to induce a streptomyces promoter to express several proteins (Murakami *et al.*, 1989, Journal of Bacteriology 171(3):1459-1466, which is hereby incorporated by reference in its entirety). Murakami *et al.* show that thiostrepton induces the expression of four proteins of unknown function in Streptomyces lividans.
30 Murakami *et al.* note that the promoter that is induced (tipA) should be useful for expression studies in streptomycetes. Since the streptomycetes are commonly used to commercially produce antibiotics, the tipA promoter can prove commercially useful. For example, certain genes can be operably linked to the thiostrepton promoter to increase their expression. These may be naturally occurring streptomyces genes (for
35 example, antibiotic producing genes) or heterologous genes (for example, mammalian genes such as interleukins, interferons, colony stimulating factors, tumor necrosis factor, etc.). (See, the two articles collected in Microbiology, 1985, American Society

for Microbiology, Washington, D.C., pages 409-420, which are both hereby incorporated by reference in their entireties.) Preferably, the improved *tipA* promoter of the present invention is linked to a DNA sequence encoding a protein so that the protein may be controllably expressed. Preferably, other DNA control sequences are included that are necessary for production of the protein, such as ribosome binding sites, etc.

As stated above, the present invention is the discovery that inserting a guanine between positions -62 and -63 of the *tipA* promoter increases the strength of the promoter while maintaining the promoter control. This is an important discovery because this promoter may be useful to controllably overexpress various proteins made in streptomycetes. Currently, streptomycetes are used for pharmaceutical and antibiotic preparations. This invention may also be advantageous in further research into promoters.

Streptomyces are gram positive organisms with a complex cycle of morphological development. They can be grown from a spore on a solid surface to give rise to a branched septate substrate mycelium and aerial mycelium (see Hopwood et al., Regulation of Gene Expression-25 Years On, The 39th Symposium of the Society for General Microbiology, Cambridge University Press 1986, pages 251-276, which is hereby incorporated by reference in its entirety). However, in submerged culture, the cells do not sporulate. After they complete the vegetative state, they remain biologically active and can produce large amounts of secondary metabolites, such as antibiotics. To illustrate this point of the 4,973 natural antibiotics described by 1,978, 2,769 were of streptomycetes origin. These antibiotics include aminoglycosides, macrolides, tetracyclines, polyethers, anasamycins, β -lactams, oligopeptides, etc. *Streptomyces* can also produce other extracellular products, such as hydrolytic enzymes. See Chater et al., 1982, *Current Topics in Microbiology and Immunology* 96:69-95, which is hereby incorporated by reference in its entirety. Although it is envisioned that the improved promoter can be inserted into all streptomycetes, exemplary hosts include the following: *Streptomyces lividans*, *S. coelicolor*, *S. griseus*, *S. parvulus*, *S. albus*, *S. vinaceus*, *S. acrimyces*, *S. calvuligerus*, *S. limosus*, *S. rubiginosus*, *S. azureus*, *S. glaucenscens*, *S. rimosus*, *S. violaceotuber*, and *S. kanamyceticus*.

As stated above, an improved promoter is important because it can be linked to a gene of interest to increase the ultimate expression of that gene. The promoter can be operably linked to genes by means known in the art (see Maniatis et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, and U.S. Serial No. 023,637, which are both herein incorporated by reference in their entireties).

Generally speaking, restriction sites may be introduced into the promoter and a specific gene of interest so that the two may be joined. Thereafter, the promoter and gene may be introduced into a vector, which may or may not be able to integrate into the streptomyces chromosome. A mechanism for inserting the promoter and/or the gene into the chromosome may be by use of a DNA sequence which favors integration. An example of this DNA sequence is the mini-circle DNA which is described below and in Lydiate *et al.*, 1986, *Mol. Gen. Genet.* 203:79-88, which is hereby incorporated by reference in its entirety.

The presently preferred *tipA* promoter differs from the wild type by one base. 10 See Figure 1 which compares the two sequences. The wild type promoter is the uninterrupted sequence, and the improved promoter is that sequence plus the guanine that is inserted between the bases at positions -62 and -63. There are also some other structures of interest. The sequences that are labeled with "a" and "b" are direct repeats. The "a" sequence is found at the positions -95 to -81, -20 to -8, and 6 to 15. 15 The "b" sequence is shown at positions -79 to -72 and -58 to -50. There are also two inverted repeats, which occur at positions -48 to -26 and -2 to -22.

Even though the most preferred embodiment is shown in Figure 1, other sequences may increase promoter strength and, therefore, transcription initiation. For 20 example, other bases may be inserted in lieu of guanine, such as thymine, adenine, or cytosine. Also, the insertion may be made at points other than between -62 and -63. The insertion could occur somewhere between -60 and -65, or even between -50 and -75. Furthermore, increased initiation may occur if the sequence shown between -55 25 and -75 on Figure 1 is shifted to a different position within the promoter. However, the shifted sequence should remain substantially homologous to the following sequence: GCGGCGGGCTCACGGGCGTGGCA (the underlining denotes the inserted guanine).

The present invention will now be illustrated by reference to the following examples, which set forth the particularly advantageous embodiments. However, it should be noted that these embodiments are illustrative and are not to be construed as a 30 restricting the invention in any way.

Example 1

Construction of an Integrated Promoter-Probe Plasmid

The integrated promoter-probe plasmid was constructed to include the following components: an *E. coli* replication origin (colE1 replication origin from the pUC18); an 35 *E. coli* selectable marker (Tn3 β -lactamase); an indicator gene (catechol dioxygenase, designated *XylE*); a *Streptomyces* selectable marker (hygromycin resistance gene); a

Streptomyces integration sequence (mini-circle DNA); and a linker region containing specific restriction sites (HindIII, KpnI, SmaI, BamHI, and XbaI). The general sequence for this plasmid's construction is shown in Figure 2 and is recited below.

The plasmid pUC18 (commercially obtained) was partially digested with PvuII 5 and then digested with EcoRI. The EcoRI restriction ends were repaired with DNA polymerases I fragment and four deoxyribonucleotide triphosphates. After the restriction ends were filled in, the plasmid was religated and transformed into E. coli DG101. Plasmids from ampicillin resistant transformants were isolated and analyzed. These plasmids were designated pEM3 (pUC18 without the lac promoter). Plasmid 10 pEM3 was ligated with DNA sequences from the plasmids pIJ2349 (hygromycin resistance) and pIJ4210 (mini-circle DNA). Plasmids pIJ4210 and pIJ2349 were obtained from John Innes Institute, Norwich NR7UH, United Kingdom. Further information can be found in Chater *et al.*, 1982, Current Topics in Microbiology and Immunology 96:69-95, for the pIJ101 series of plasmids and Hopwood *et al.*, Genetic Manipulation of Streptomyces, a laboratory manual, The John Innes Foundation, 15 Norwich, United Kingdom. The hygromycin resistance gene was obtained by digesting pIJ2349 to obtain a 1.0 kb fragment. The mini-circle DNA was obtained by digesting pIJ4210 to obtain a 2.10 kb fragment (see Lydiate *et al.* referred to above). After digestion with EcoRI and PstI, pEM3 was joined to the fragments from pIJ2349 20 and pIJ4210 in a three fragment ligation to yield pHC-I. Plasmid pIJ4081 was obtained from the John Innes Institute and was digested as shown in Figure 2. After digestion with restriction enzymes, a 1.5 kb fragment containing the XylE and the linker region was obtained and ligated to the fragment generated from pHC-I, as also shown in Figure 2. The resulting plasmid was designated pHC-2.

25

Example 2

Cloning of the tipA Promoter

The tipA promoter was constructed using two overlapping oligonucleotides, a 77 and 78 base primer, as shown as follows:

77-mer CCAAGCTTCC CGGGCTGAGG GAGCCGACGG CACCGGGCGG CTCACGGCGT GGCACGGCGGA

30

ACGTCCCCGC TTGCACC

78-mer CCTCTAGACT TCCGCTCCCT TCTCTGACGC CGTCCACGCT GCCTCCTCAC GTGACGTGAG

GTCGAAGCCC GGACGTTTC

35

These two primers were annealed and extended by DNA polymerase I large fragment and deoxyribonucleotides. After extension, the double-stranded DNA was digested with HindIII and XbaI. The digested DNA was cloned into the HindIII-XbaI site of pUC18. The recombinant plasmid was sequenced. It contained the improved promoter

having the guanine insertion between bases -62 and -63. The promoter was designated *tipA*-VG62. The wild type and mutant promoters were subcloned into the promoter-probe plasmid pHG-2. The plasmid having the wild type promoter was designated pHG11-11, and the plasmid having the improved promoter was designated pHG11-3.

5

Example 3

tipA Promoter Activity and *Streptomyces lividans*

Both plasmids were then transformed into *Streptomyces lividans*. Cultures of *Streptomyces lividans* were grown and the specific activity of the *XylE* gene was analyzed in the presence and absence of thiostrepton (thiostrepton induces the *tipA* promoter to transcribe the *XylE* gene). The results of this experiment are shown in 10 Table I.

Table I

tipA Promoter Activity in *Streptomyces lividans*

Construct	Promoter	Specific Activity of <i>XylE</i> *	
		-Thiostrepton	+Thiostrepton
pHG11-11	<i>tipA</i> -wt	< 0.5	36.3
pHG11-3	<i>tipA</i> -VG62	12.0	182.2

* Specific activity is defined as mmoles of catechol semialdehyde formed per minute per mg of protein.

20

The results show that there was 5-fold more protein produced from the plasmid having the improved promoter.

The plasmid pHG11-3 has been deposited in Cetus' in-house depository as CMCC 3798 and in the ATCC as 40737.

25

The present invention has been described with reference to specific embodiments. However, this application is intended to cover those changes and substitutions which may be made by those skilled in art without departing from the spirit and the scope of the appended claims.

In the Claims

1. A DNA sequence comprising a Streptomyces promoter, wherein a base is inserted into the base sequence between positions -50 and -75, to increase transcription initiation.
- 5 2. A DNA sequence in accordance with Claim 1, wherein a base is inserted into the base sequence between positions -60 and -65, to increase transcription initiation.
3. A DNA sequence comprising a Streptomyces promoter, wherein a guanine is inserted into the base sequence between positions -62 and -63.
- 10 4. A DNA sequence in accordance with Claim 3, wherein the DNA sequence is regulated by thiostrepton.
5. A DNA sequence that hybridizes to the following DNA sequence: GCGGCGGCTCACGGCGTGGCA and which increases transcription initiation when placed upstream of a gene.
- 15 6. A DNA sequence in accordance with Claim 5 comprising the base sequence shown in Figure 1, from positions -1 to -100, including the guanine insertion between -62 and -63.
7. A DNA sequence in accordance with Claim 6 further comprising operably linking a gene to the DNA sequence to control the expression of the gene.
- 20 8. A DNA sequence in accordance with Claim 7, wherein the promoter and gene are contained in an expression vector.
9. A DNA sequence in accordance with Claim 7, wherein the promoter and gene are contained in the chromosome.
- 25 10. A DNA sequence in accordance with Claim 7, wherein the gene is a heterologous gene.

11. A DNA sequence in accordance with Claim 10, wherein the gene encodes for a protein selected from the group consisting essentially of interleukins, interferons, colony stimulating factors, and tumor necrosis factor.

12. A DNA sequence in accordance with Claim 7, wherein the gene is found in
5 Streptomyces.

13. A DNA sequence in accordance with Claim 12, wherein the gene encodes for a protein selected from the group of antibiotics consisting essentially of aminoglycosides, macrolides, tetracyclines, polyethers, anasamycins, β -lactams, and oligopeptides.

10 14. A method for producing a protein comprising operably linking a gene encoding the protein to the DNA sequence of Claim 1 and expressing the protein.

15. A method for producing a protein comprising operably linking a gene encoding the protein to the DNA sequence of Claim 3 and expressing the protein.

15 16. A method for producing a protein comprising operably linking a gene encoding the protein to the DNA sequence of Claim 6 and expressing the protein.

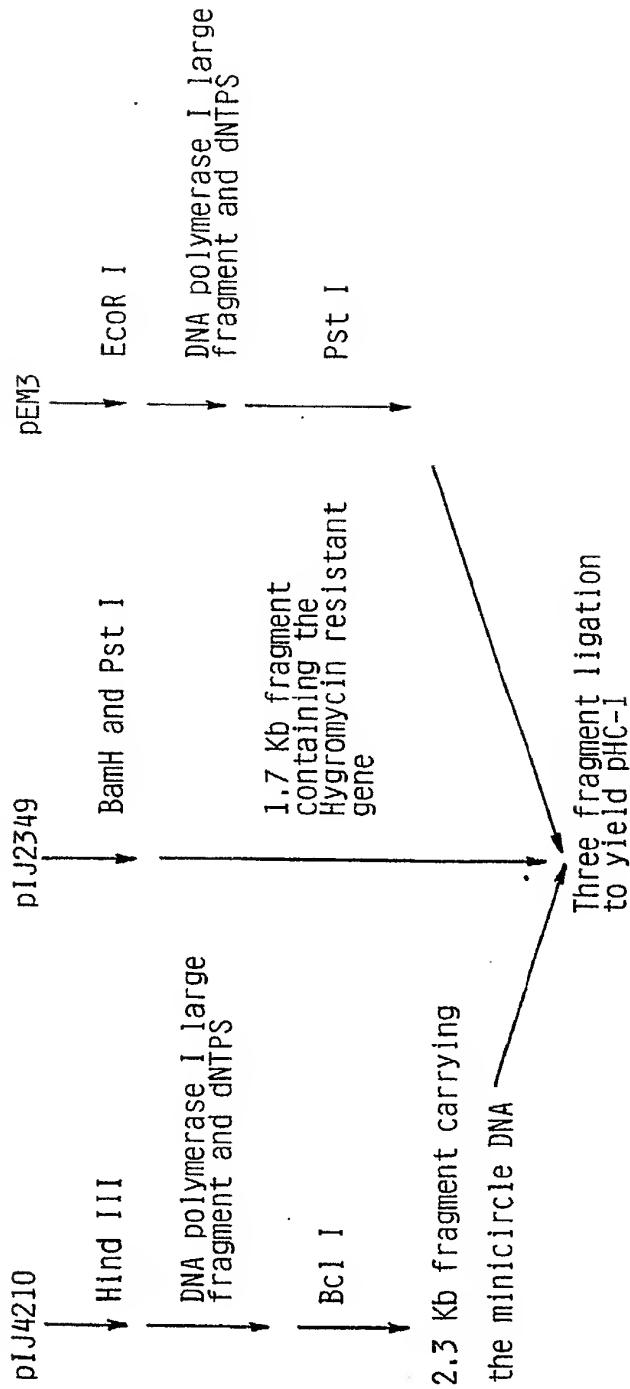
DNA sequence of the *tipA* wild type and mutant promoters

HindIII -100 -90 -80 -70 G -60 -50
AAGCTTCCGGGCTGAGGGAGCCGACGGCACGCGGCGGCTCACGGCGTGGCACGC
 a b b
 -40 -30 -20 -10 *
AACGTCCGGGCTTGCACCTCACGTACGTGAGGGAGCGTGGACGGCGTCAGAGA
 a
AGGGAGCGGAAGTCTAGA
 a XbaI

FIG. 1

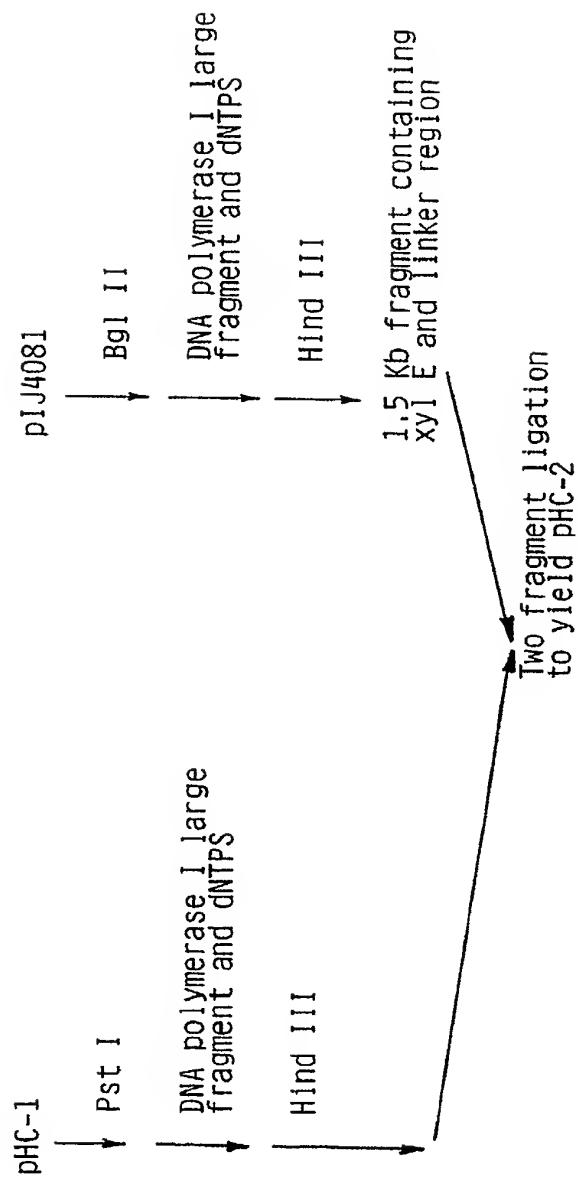
1. CONSTRUCTION OF pHc-1

FIG. 2-1

**SUBSTITUTE SHEET**

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2. Construction of pHc-2



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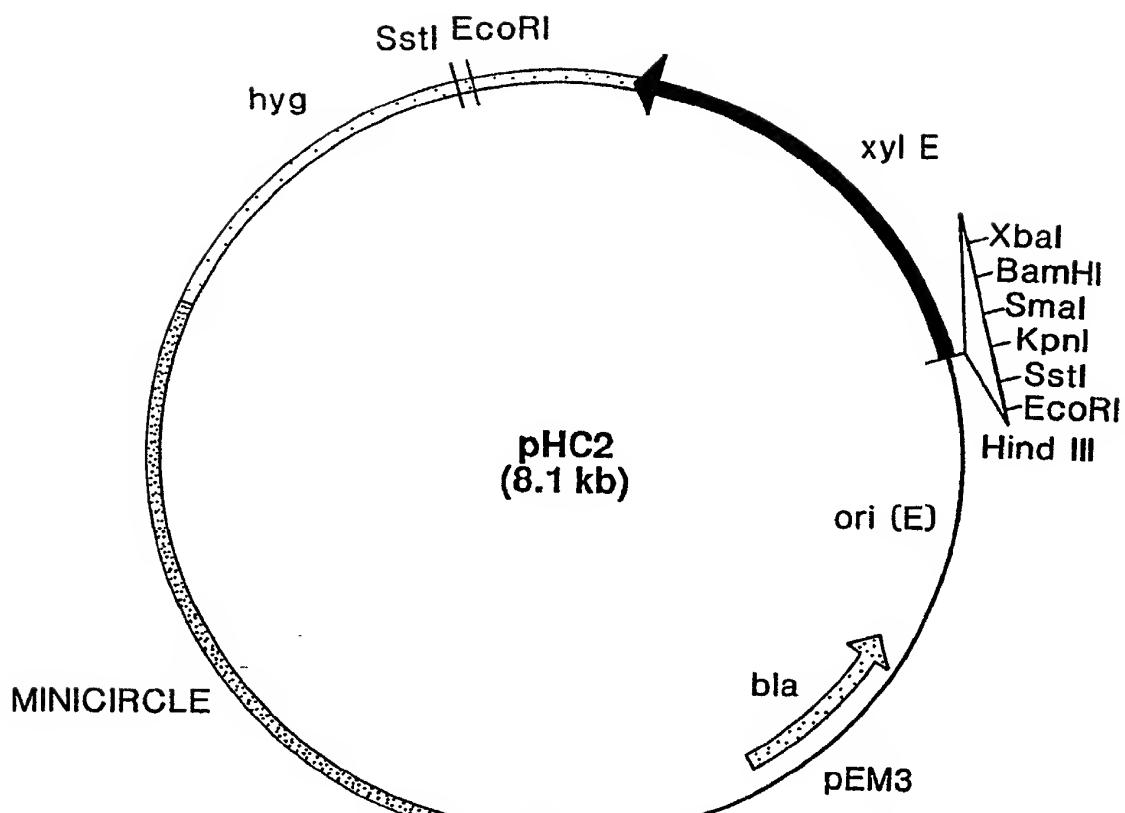


FIG. 3

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 90/07348

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 12 N 15/76		
II. FIELDS SEARCHED Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC5	C 12 N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	<p>JOURNAL OF BACTERIOLOGY, vol. 171, No. 3, 1989, T. Murakami et al: "Thiostrepton-Induced Gene Expression in Streptomyces lividans", see page 1459 - page 1466</p> <p>-----</p> <p>-----</p>	1-16
<p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
5th April 1991	10.05.91	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	 Alfredo Prein	

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/US 90/07348

SA 43377

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date

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